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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of: VLADIMIR KONSTANTINOVICH SOLOGUB

Serial No. US No. 09/890,496

Filing date: July 31, 2001

For: USE OF POLYACRYLAMIDE GEL FOR FORMING A CAPSULE IN THE TISSUE OF THE ORGANISM OF A MAMMAL, AND A METHOD OF CULTIVATING CELLS, AND A METHOD OF TREATING ONCOLOGICAL DISEASES AND DIABETES MELLITUS

DECLARATION UNDER 37 C.F.R. § 1.132

1. I, the undersigned, Vladimir Konstantinovich Sologub, hereby declare that I:

2. am Russian citizen, resident of Moscow, ul. Garribaldi, 10, corp. 3, apt, 304.

3. graduated from Moscow Veterinary Academy in 1971.

4. am employed in Moscow Research Institute of Medical Ecology, and presently hold the position of a head of laboratory.

5. duration of my practical work experience in the field of immunology and cellular biology: from 1971 up to the present time. For the work in this field I have been conferred the degree of candidate of biological sciences (PhD).

6. am a co-inventor of the invention disclosed in US Application N09/890,496 titled "Use of polyacrylamide gel for forming a capsule in the tissue of the organism of a mammal, and a method of cultivating cells, and a method of treating oncological diseases and diabetes mellitus".

7. have conducted experimental investigations on formation of a connective-tissue capsule in organism of a mammal by insertion of polyacrylamide gel into tissue of a mammal, and on a possibility to implant the allo- and xenogenic cells into said capsule and a possibility to maintain viability of said cells. Findings of said investigations have demonstrated that the allo- and xenogenic cells, having been introduced into said formed capsule, retain their viability and proliferative activity as long as three months.

For that purpose I used two groups of animals: Wistar-breed rats and August-breed rats, 10 animals in each of the groups. 5 females and 5 male were kept in cages, 5 animals in each of the cages, who received a standard feed and abode under the following constant environmental conditions: at temperature of 18-25°C, humidity of 65-80%, daylight hours – 128. After two-week adaptation of said rats obtained from "Stolbovaya" laboratory animal farm of Russian Academy of Medical Science (RAMS), 2 ml of polyacrylamide gel (PAAG) was administered subcutaneously in the area of right scapula to them. The animals were being observed daily to

note the general responses to the inserted material, local condition of integuments, presence/absence of appetite and motion activity. Two-month observation did not detect any deviations from the applicable norms in the tested animals. PAAG administered to the rats was remaining within the insertion locus in the form of a subcutaneous formation that did not affect a condition of the hair-covering and skin. The insertion loci did not exhibit any oedemas and inflammatory responses. After two months the tested animals were sacrificed by placing them in the environment of exiccator-ether-air mixture for 40 minutes. Autopsy of the animals showed that polyacrylamide gel under animals' skin was surrounded by a thin vascularized connective-tissue capsule. In the surrounding tissues, any signs of inflammation and/or infiltration by lymphoid cells were not detected. The connective-tissue capsules together with the contiguous tissues were placed in 10% neutral formalin solution for further histological study. Any deviations in morphology of internal organs in the tested animals have not been found.

In another series of the conducted experiments I used mice of BABB/c, "C57Black/6 lines of hybrids F_1, Balb/c x DBA/2 obtained from "Stolbovaya" farm of RAMS. Groups of 10 females of each of the lines and hybrids were kept in cages under the standard conditions and used in the contemplated experiments after their 2-week adaptation. 4% PAAG was administered subcutaneously in the area of right scapula, in the amount of 0,5 – 1,0 ml per mouse. The animals were observed daily to register any deviations and their appearance. 2 weeks of said observation did not find any convincing deviations from the applicable norms in the tested animals. Polyacrylamide gel was remaining in the insertion loci, causing no irritation or inflammatory responses, and, being palpated, was felt as a soft elastic matter that was not changing its location under skin of the animals. After 2 months subsequent to the beginning of the experiment, 2 mice of each of the groups were euthanised by placement in an exiccator filled with ether vapours for 40 min. Autopsy of the tested animals showed that PAAG under skin of the mice was disposed within a thin vascularized connective-tissue capsule. Any signs of inflammatory responses, lymphoid infiltration and/or oedemas and fibrosis were not detected. Any deviations in the tissues and internal organs surrounding the gel were not detected.

7.1 I carried out transplantation of the allogenic cells into a connective-tissue capsule filled with polyacrylamide gel after 4 – 8 weeks the gel had been inserted, under the following conditions. The following transplantable tumours were used as the allogenic transplants for mice: melanoma B16 and adenocarcinoma Ca-755 maintained in syngeneic mice of C57Black/6 line. The cells were transplanted as the solid subcutaneous tumours which had developed after 5 – 12 days after administration of 1 mln. cells into syngeneic mice C57Black/6. The transplanted tumours' cells were obtained from RAMS Oncological Centre and stored in nitrogen 10% DMSO and 50% FBS as cryopreservatives. The recipients were the Balb/c-line mice, to each of

whom, 2 months prior to the experiment, 1 ml of polyacrylamide gel was subcutaneously administered. 1 mln tumour cells of melanoma B-16 and adenocarcinoma Ca-755 were inserted into PAAG in the amount of 0.2 ml; 4 animals per tumour. Growth of a tumour within the gel was detected after 5 – 7 days by palpation of the gel.

The allogenic tumours, in the case of transplantation into a connective-tissue capsule with the gel, grew sufficiently rapidly, in 30-40 days having reached the dimensions comparable with those of recipient mice themselves. In contrast with the syngeneic growth, the allogenic tumours did not produce any metastases, nor necrotized for 40 days (the monitoring period). In the end of the experiment the animals were sacrificed in an exiccator filled with ether vapours. It should be noted that the allogenic tumours per se had not been the cause of which the tested animals perished – despite their very large sizes. Autopsy showed the absence of metastases in internal organs, enlarged spleen and liver, lymphoid infiltration in the connective tissue contiguous to a tumor. Growth of B-16 and Ca-75 tumours in non-syngeneic mice of Balb/c-line is shown in Figs. 1 and 2.

The allogenic hybridomas. The murine hybridomas that produce, in Balb/c-line mice, the tumours being monoclonal to viral antigens and transplantable as the ascetic ones, were introduced into gel-capsules inserted in the non-syngeneic C57Black/6-line mice. To determine production of antibodies by the hybridoma cells placed in the gel-capsule, blood samples from the tested mice' tail vein was taken once a week. Antibodies were determined by the standard immunoenzyme method using a specific viral antigen and caprine peroxidase conjugate of anti-IgC mice. Monoclonal antibodies were being determined in the tested mice serums during 4 weeks. All that time the solid tumours formed by the allogenic hybridoma were palped in the gel under the mice' skin. A reduced production of antibodies in the end of the experiment coincided with an increased mass of the allogenic tumour implanted into the gel.

7.2 Transplantation of xenogenic cells into a connective-tissue capsule filled with polyacrylamide gel was carried out by me as follows. The human and murine xenogenic tumour cells in PAAG in mice and rats. Tumour lines of human cells, SKMEL-1 melanoma, and MCF-7 and LNCap adenocarcinoma were obtained from American Tissue Type Collection (ATTC) and cultivated "in vitro" in accordance with the provider's recommendations in RPMI medium with 10% FBS. Murine B-16 melanoma was used as the xenogenic transplant for rats. Before their insertion into the gel, the cells were concentrated by low-speed centrifuging, and PAAG was administered to animals subcutaneously in the amount of 0.5 – 1.0 mln in 0.2 ml of a growth medium. The animals were being observed daily for 3 – 4 months to register their general condition and local responses in the gel and cells introduction locus. No general or local responses in the animals were detected in the first days after the xenogenic tumour cells had been

inserted into the gel. After 7 – 10 days, solid tumours started to be felt by palpation in the gel, and said tumours gradually filled the entire volume of the gel. The human tumour cells were extracted from mice and rats after 1, 2 and 3 months after the experiment commenced by placing the aseptic extracted gel fragments with cell islets in a growth nutrient medium and cultivating them in environment of CO_2 in an incubator at 37°C. The tumour cells, under the "in vitro" conditions, returned to their initial morphology and were cultivated in the same manner as the cell culture prior to insertion into the gel.

7.3. I also used the normal human, pig and rabbit cells for inserting them into a capsule previously formed in mice and rats. The human thyroid cells (an operation material) were collagenized and placed in the nutrient RPMI-1640 medium with 10% FBS. After 7 days of cultivation, when the living thyroid gland cells were represented by both the differentiated and differentiated-blast cells, they were inserted into PAAG in BALB/c mice. In all cases, vascularization of the connective-tissue membrane was enhancing, and the living xenogenic cells were observed in the animals sacrificed and autopsied on 30 – 60 days after commencement of the experiment.

The normal cells of Langerhance islets were isolated from pancreases of new-born rabbits and pigs using type-4 collagenase (Gibco). The islets were cultivated "in vitro" for 7 – 14 days (2 – 3 passages) and inserted into PAAG that had been pre-implanted subcutaneously in rats, the dose being 250 – 300 th. cells in 0.2 ml of the medium (Fig. 3). Blood samples were taken once a week from the tail vein of the animals, and, in serum, concentration of glucose (photo-glucose set) and presence of the rabbit and pig insulins (IFA on the antibodies monoclonal to the porcine insulin) were determined. The xenogenic insulin was detected in the rat blood serum on 7 – 20 days after the pancreas cells were grafted. Presence of an additional insulin did not affect the glucose level, and in all tested animals said insulin persisted to be at the normal level. In the course of additional experiments I studied a possibility to correct STZ – the induced diabetes in rats – by way of administration of the rabbit β-cells for diseased animals into a preformed connective-tissue capsule. Using the above-described technique, the glucose content in the tested animals' blood serum was successfully reset to the norm in all cases. Results of said experiments, for 4 animals, are shown in Fig. 4.

8. I scrutinized Lamberti's invention (US patent 5,827,707). Due to lack of required time and necessary equipment I was not in a position to reproduce the experiments conducted according to said reference. However, as regards the technique for incapsulating the cells in PAAG and application of such incapsulated cells for treatment of diabetes mellitus in particular, the approach described by Lamberti substantially differs from the technique described in the Application of my co-inventorship. The most important differences are recited in Table 1.

Table 1.

Parameter	**Lamberti (US 5,827,707)**	**Zybin et al. (US App. 09/980,496)**
Location of capsule formation (LCF)	Ex vivo	In tissue of a mammal
Intracapsule microenvironment at the moment of inserting of cells	Components of a synthetic gel at the polymerization stage	Polymerized gel, balanced with organism physiological liquids in respect of content of nutrients and growth factors
Laboratory animals, in which efficacy of the method in treatment of diabetes mellitus was demonstrated	Athymic mice, deprived of the graft immonorejection capacity	Healthy rats, having a sound immunity
Results of treatment of human diabetes mellitus	none	Examples cited in the Application

One may infer from comparison of said two techniques that though Lamberti's invention was published much earlier, our Application comprises essentially the novel teachings and attained results as compared with said invention.

First, in our case, a capsule is formed not outside, but inside the mammal organism. Second, the cells are inserted into the gel subsequent to a certain period after the gel has been administered to a mammal, which period is sufficient for formation of a continuous connective-tissue capsule around the gel. It is exactly said capsule that performs the main function of protecting the allo- and xenogenic cells transplanted into the gel. Further, in the course of formation of the capsule (4 – 8 weeks), PAAG is saturated to the physiological concentration by the host organism's nutrient and growth factors. For this reason, the cells inserted into such capsule, immediately enter the medium that is favourable for their vital activity and growth.

The microcapsules provided according to Lamberti's technique lack the above-mentioned advantages. The cells included into capsules at the cell polymerization stage from the very start are under worse conditions than in our case. In Lamberti's technique, a capsule having no connective-tissue shell is less protected against the immune reaction educed by the host organism. According to the example that shows the functional *in vivo* activity of the xenogenic islet pancreas cells, Lamberti had to use the athymic mice. Otherwise he might not be able to demonstrate the positive result - due to a rapid immunorejection of the transplanted cells. The example cited by me of a long-term functional activity of the rabbit β-cells inserted into the pre-formed connective-tissue capsule in healthy rats, clearly demonstrates protectability of the xenogenic cells transplanted according to our technique, thereby attesting to an advantage thereof.

Lamberti in his publication merely suggests a possibility of using his technique to treat the human diabetes mellitus. The examples based on case reports and cited in our Application do explicitly prove efficacy of the method we claim.

9. I have scrutinized Chaikof's works (Annual Review of Biomedical Engineering, 1999, vol. 1, pp. 103-127) and those done by Sefton and Stevenson (Advances in Polymer Science, 1993, vol. 107, pp. 143-197). Said references describe in detail the methods allowing to enclose the living cells within macro- and microcapsules formed by different polymeric materials. Chaikof substantiates characteristics of the polymeric membranes capable of ensuring viability of the transplanted cells enclosed therein, he also recites techniques for providing such membranes. Sefton and Stevenson describe different mechanisms employed for microincapsulation of cells with use of polymers and combined polymers, and expound morphology of the obtained capsules and their applicability for cell transplantation. The used materials and techniques for obtaining the polymeric capsules, recited in said references, being rather diverse, in these references, similarly to Lamberti's patent, the following general approach to the matter of cell transplantation persists, which is: incapsulation of the cells *in vitro* and subsequent insertion of the cells protected in such manner into the recipient's organism. Though all above-mentioned publications described the use of PAAG in its capacity of a protective material, inclusive of β-cells that produce insulin, their technique for using PAAG for said purposes substantially differs from the technique proposed in our application. The main differences of the techniques are outlined in Table 2.

TABLE 2.

	1	2	3	4	5
	Zybin et al.	Lamberti	Chaikof	Sefton and Stevenson	Combination of 2, 3, 4
Location of formation of capsule (LFC)	In tissue of a mammal	Ex vivo	Ex vivo	Ex vivo	Ex vivo
A method and time for inserting cells into gel	Injection into gel after 4 – 8 weeks after the gel has been inserted into an organism	Adding into a reaction mixture at the polymerization moment			Adding into a reaction mixture at the polymerization moment
The factor that ensures protection of the transplanted cells	A naturally formed capsule of a connective tissue	A PAAG layer	Modified polymeric membrane having a controllable porosity and permeability		Artificial insulating layer of a polymer

Capsule stability	Guaranteed integrity of a depot that comprises the transplanted cells, which integrity is ensured by a continuous connective-tissue layer surrounding a depot	1. A possibility of a mechanical damage or destruction of microcapsules in time of insertion into organism, resulting in a leak of an antigen material and in an accelerated development of the immune response to a transplant. 2. A possibility of infiltration of the immune cells and soluble immune factors into a capsule.		A risk of disruption of capsule integrity, development of the host's immune reaction, and of elimination of a transplant.
The transplanted cells' life-sustenance factor	1. Preliminary saturation of gel by nutrients and growth factors so that the physiological concentration will be reached 2. Vascularization of connective-tissue capsule	Diffusion of nutrients in a gel capsule, subsequent to insertion into organism	Pervasion of low-molecular substances into a capsule, caused by properties of an external polymeric membrane	1. Absence of a "starting" stock of nutrients in a capsule. 2. Absence of "active" provision of a capsule with the vascular system.

Thus, it may be concluded that our invention has the features that could not be created by a person skilled in the art basing on the results adduced in the references of Lamberti, Chaikof, Sefton and Stevenson, said features being not a combination of those described in said publications.

10. The undersigned further declares that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

Executed on this 18 th day of May 2004

VLADIMIR KONSTANTINOVICH SOLOGUB

Vladimir Konstantinovich Sologub

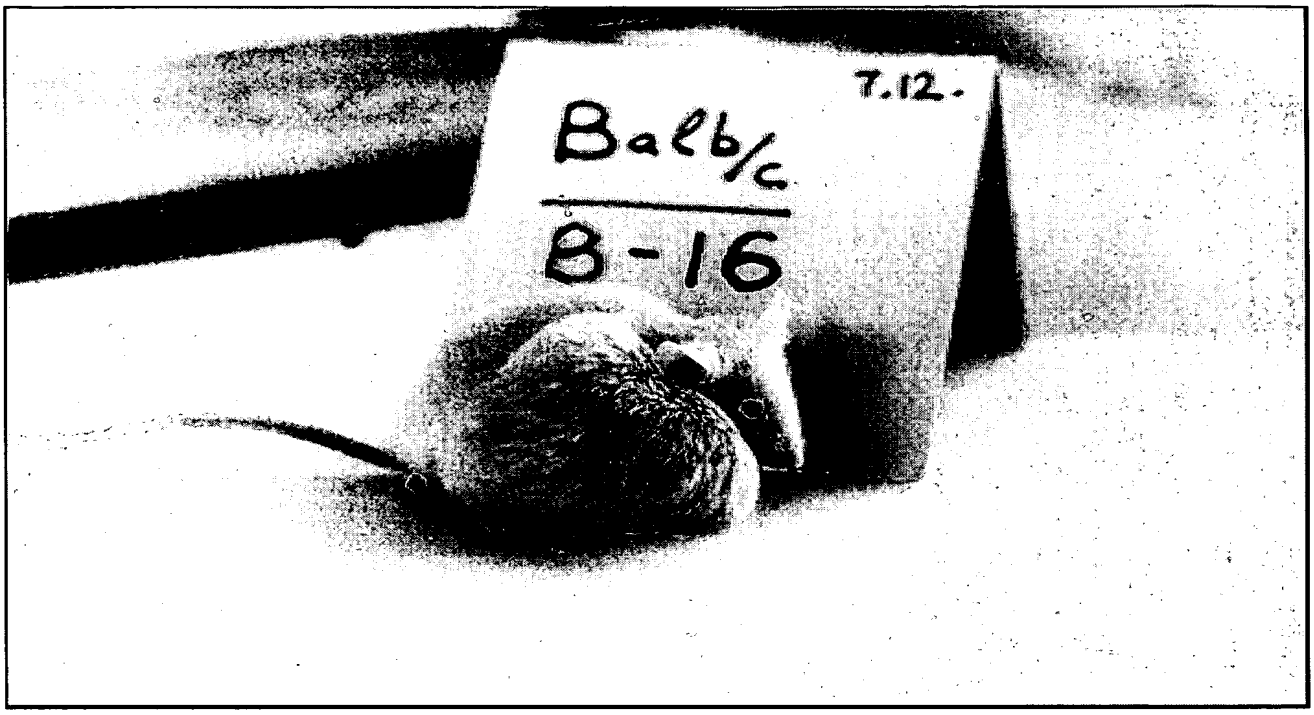

Fig.1. Growth of B-16 melanoma tumor in gel bearing Balb/c mice

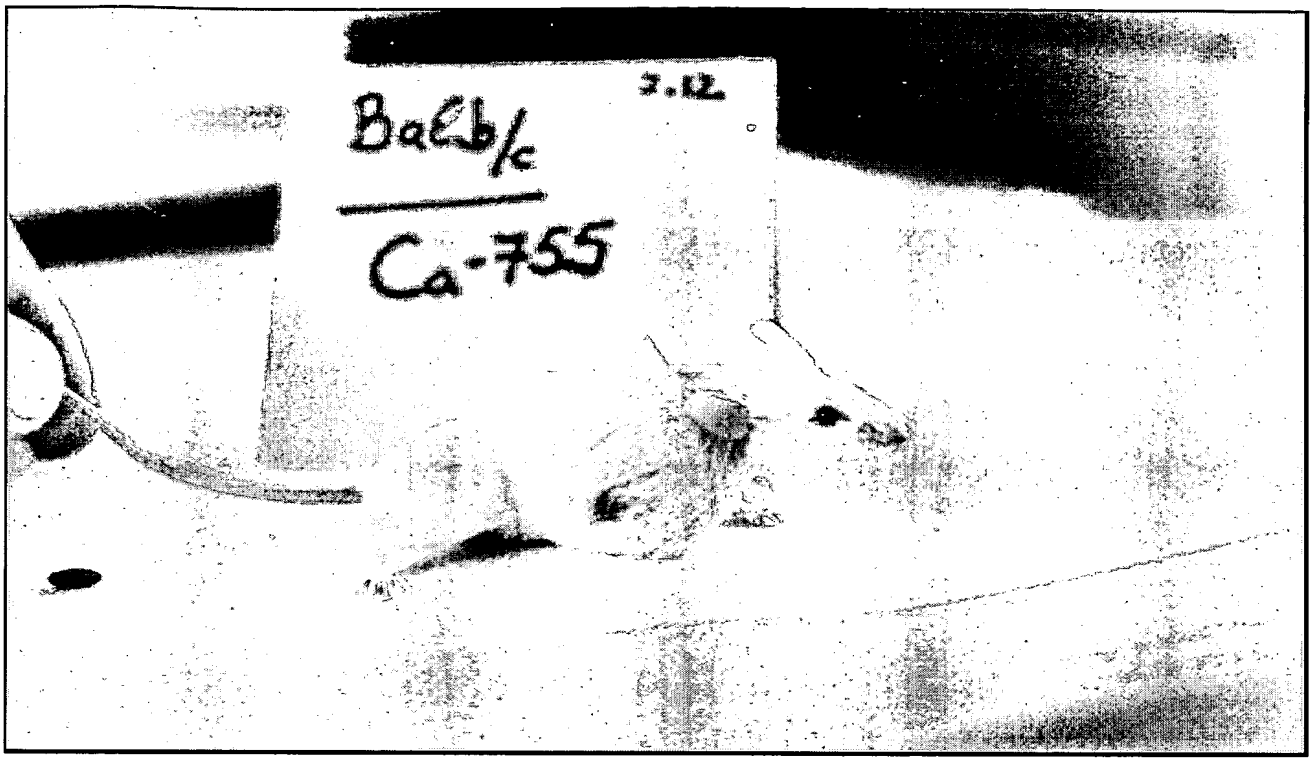

Fig.2. Growth of Ca-755 mammary carcinoma in gel bearing Balb/c mice

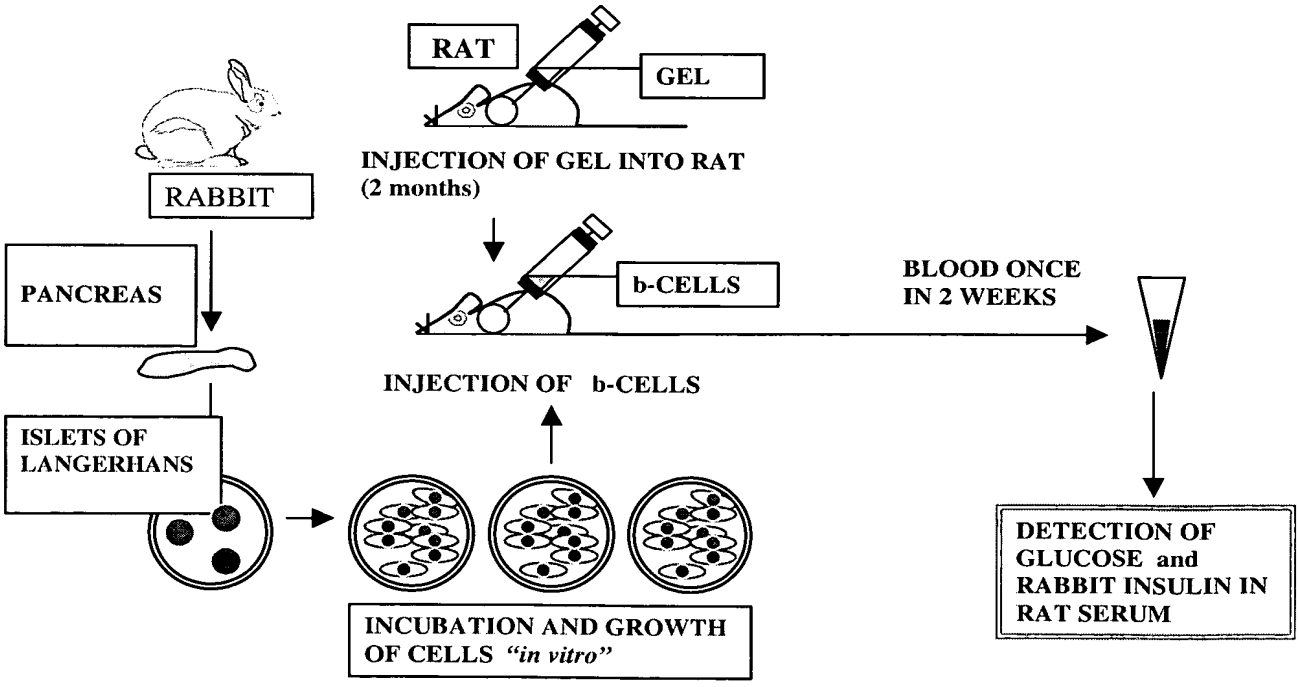

Fig. 3. SCHEME OF EXPERIMENT

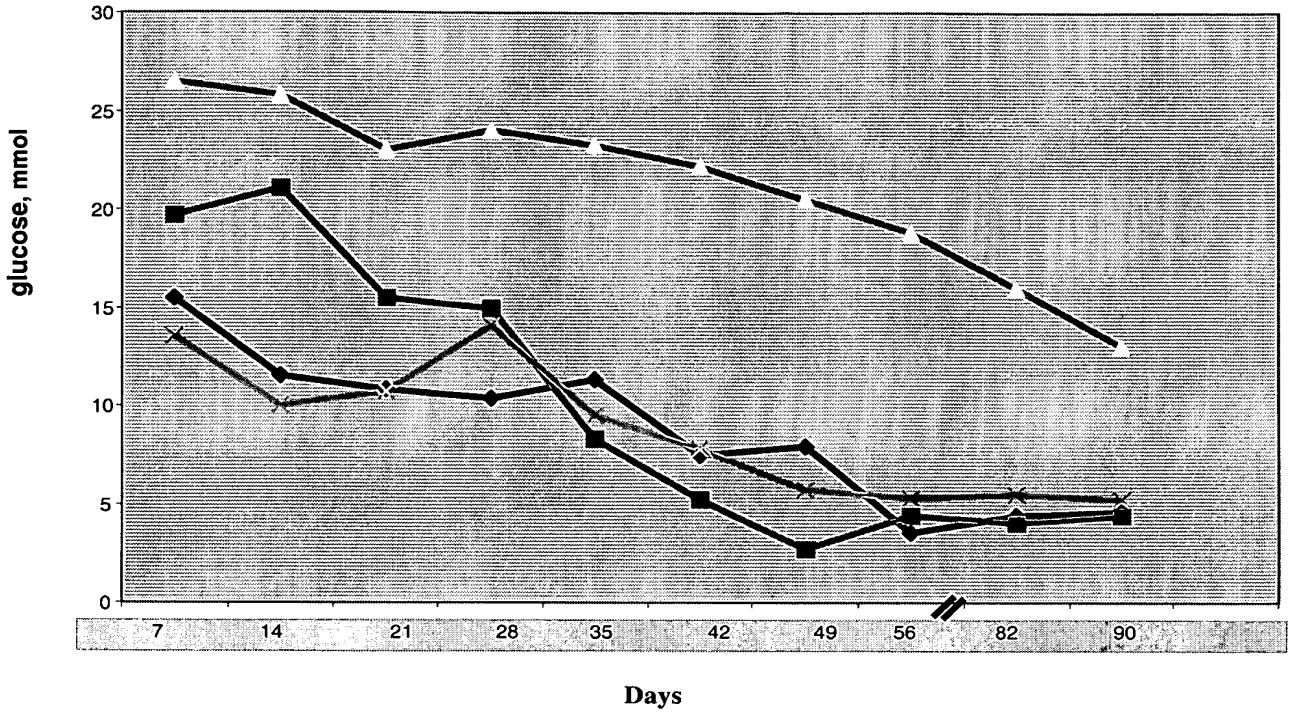

Fig.4. The glucose level of experimental rats after subcutaneous injection of 50 mg/kg of streptozotocin with implanted rabbit beta-cells